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The Estimation of D- and L-Glutamic Acid in Proteins

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In connexion with studies on the glutamic acid content of tumour proteins it was necessary to estimate the respective amounts of the two optical isomers given on acid hydrolysis. The enzymic decarboxylation procedure of Gale (1945) was applicable to the L-isomer, but a search for a specific method for the D-isomer was not successful. Experience showed that it was not oxidized by the specific D-amino acid oxidase of kidney and that the activity of fungal oxidases (cf. Bender & Krebs, 1950; Emerson, Puziss & Knight, 1950) was very low. Moreover, the preparation from rabbit liver of an enzyme able to oxidize D-glutamic acid and D-aspartic acid (Still, Buell, Knox & Green, 1949; Still & Sperling, 1950) could not be repeated. Total glutamic acid was therefore estimated by an ion-exchange method (Consden, Gordon & Martin, 1948), which does not distinguish between the isomers, and the D-component found by difference. The present communication concerns the application of this two-step procedure to four purified proteins as well as to certain protein preparations and derivatives which have been reported by previous workers to contain a fair proportion of the D-isomer. In addition, opportunity is taken to present new evidence on the racemization of the L-isomer during acid hydrolysis.

EXPERIMENTAL

General methods

Nitrogen determinations were made by the micro-Kjeldahl procedure. Amino N was estimated by the method of Van Slyke (4 min. reaction time). L-Glutamic acid was estimated by the decarboxylase procedure of Krebs (1948), using a washed suspension of *Clostridium welchii* S.R. 12. Optical rotations were determined in a 4 dm. tube unless otherwise stated. Protein hydrolysates were prepared by boiling with 6N-HCl for 24 hr. Evaporations *in vacuo* were conducted below 40°.

Materials

The sample of L-glutamic acid hydrochloride used was recrystallized; $[\alpha]_D^{20} + 31{\cdot}4°$ (*c*, 4·0 in 2·5N-HCl). The other amino-acids were purified commercial samples. The following proteins were purified samples available in the laboratory; the N content is quoted on a moisture- and ash-free basis unless stated: Edestin, N = 18·65%; ox insulin, recrystallized according to Du Vigneaud, Miller & Bodden (1939), N = 15·58%; horse myoglobin, N = 16·6% (not corrected for ash). Preparations of casein and gelatin described below were made from commercial samples.

L-Pyrrolidonecarboxylic acid. L-Glutamic acid was heated as described by Wilson & Cannan (1937). The anhydride was extracted from aqueous solution with ethyl acetate, from which solvent it was twice recrystallized. $[\alpha]_D^{20} - 9{\cdot}92°$ (*c*, 7·5 in water).

Amino acid control mixtures. These were made up to simulate *A*, haemoglobin; *B*, gliadin; *C*, myoglobin (Table 1). *A* was rich in aspartic acid, *B* in glutamic acid and *C* had these two amino acids present in the proportions usually found in proteins.

Tobacco-mosaic-virus protein. This was prepared by heat coagulation of the nucleoprotein and was a gift from Dr J. D. Smith, Molteno Institute, Cambridge; N = 17·89% (not corrected for ash).

Dried cells of Lactobacillus casei. Strain 3253 of the National Collection of Type Cultures was grown for 50 hr. at 30° in 1 l. flasks of the medium described by Rodwell (1953). The flasks were chilled to 0° and the bacteria separated by centrifuging. They were washed thrice with

M-NaCl, once with 50% (v/v) aqueous ethanol, twice with 98% (v/v) ethanol, thrice with ether and then dried over P_2O_5 at 5 mm. Hg and room temperature. The weight of dried cells from 3 l. of culture was 2·78 g. (N = 10·03%).

Table 1. *Composition of amino-acid control mixtures*

Amino acid	Weight (mg.) A	B	C
DL-Alanine	295·7	23·8	44·2
DL-Valine	318·0	29·8	37·4
DL-Leucine	398·9	33·2	170·9
DL-Isoleucine	238·3	—	—
DL-Serine	230·3	62·2	22·7
DL-Threonine	174·0	—	59·7
L-Proline	156·2	150·6	23·0
DL-Tyrosine	207·6	34·3	22·5
DL-Phenylalanine	317·7	72·2	75·4
DL-Methionine	36·5	18·8	16·8
L-Cystine	40·8	29·7	—
L-Tryptophan	38·3	8·5	33·6
DL-Lysine hydrochloride	382·4	15·1	198·6
L-Histidine	—	21·0	108·7
L-Histidine dihydrochloride	464·5	—	—
L-Arginine hydrochloride	179·5	38·0	31·2
L-Aspartic acid	708·5	18·3	125·6
L-Glutamic acid hydrochloride	485·5	647·7	188·3
Ammonium chloride	121·7	294·1	—

Racemized products from casein. Dakin & Dudley (1913) reported that the glutamic acid of casein was completely racemized by dilute alkali at low temperature. 50·27 g. casein (air-dry), 500 ml. 0·5N-NaOH and a few drops of toluene were placed in a 1 l. flask, the mixture shaken and then incubated at 37° for 23 days, shaking at intervals. The mixture was then filtered, the filtrate neutralized with H_2SO_4 and adjusted to 1 l. Glacial acetic acid was added to precipitate the 'racemized casein', which was then filtered, washed and dried. The collected filtrate and washings are referred to in Table 6 as 'racemized caseose'.

Racemized products from gelatin. 50 g. gelatin (air-dry) were treated as described by Dakin (1912). The incubation mixture was separated by dialysis in cellophan into an indiffusible 'protein' fraction and a diffusible 'peptide' fraction (Table 6).

Estimation of glutamic acid by means of Amberlite IR-4B

The procedure followed closely that of Consden *et al.* (1948): Amberlite IR-4B resin was stirred and washed by decantation with water or dilute HCl until the pH of the supernatant liquid reached and remained at the required value. A portion of the hydrolysate (10–20 mg. N) was placed on a 1 cm. diam. column containing 5 g. Amberlite IR-4B equilibrated to pH 3·4. The column was washed with 200 ml. water and the washings discarded. The dicarboxylic acids were eluted with 100 ml. N-HCl and the eluate taken to dryness repeatedly *in vacuo* to remove excess mineral acid. The residual hydrochlorides were dissolved in a minimum volume of water and transferred to a similar column equilibrated to pH 2·6. This column was eluted with 0·001N-HCl, amino N being determined on successive 5 ml. fractions of the eluate. Under these conditions glutamic acid was eluted before, and distinctly separated from, the aspartic acid. The first column had to be prepared fresh for each determination, but the second, which maintained its pH indefinitely, could be used for several runs. After the first run the appearance of the glutamic acid and aspartic acid could be predicted in replicate analyses on the same column. The fractions containing glutamic acid were pooled, and amino N estimated on a sample. The purity of the glutamic acid was established by paper chromatography. Although no other amino acid was ever present as a contaminant in the fractions containing the dicarboxylic amino acids, there was a small quantity of aromatic amino acids eluted ahead of the glutamic acid. The eluate also contained traces of resin-depolymerization products which reacted with ninhydrin so that it was not possible to use the quantitative colorimetric method of Moore & Stein (1948) to estimate the glutamic acid. A small correction (2 μg. amino N/ml. eluate) was made for resin breakdown.

Racemization of L-glutamic acid under the conditions of protein hydrolysis

The extent of racemization was determined by polarimetry. To give maximum observed rotation a 4 dm. tube was used, which permitted a reading of about +5° to ±0·02° or ±0·4%. The results of a series of experiments are listed in Table 4: each recorded rotation represents the mean of twenty readings. In treatments (*b*) and (*c*) the glutamic acid hydrochloride was placed in a 500 ml. flask, 100 ml. 6N-HCl were added and the solution taken to dryness *in vacuo* either once (*b*) or several times (*c*) as is customary when dealing with protein hydrolysates. The residue was redissolved in 2·5N-HCl for polarimetry. In treatments (*d*) and (*e*) the hydrochloride was boiled under reflux for 24 hr. before taking to dryness as in (*b*) and (*c*) respectively.

Optical form of glutamic acid in control experiments

The recovery of glutamic acid on a column of Amberlite IR-4B was satisfactory (Table 2). Using the decarboxylase procedure the recovery of L-glutamic acid was not significantly different (Table 3), except when the control mixture was submitted to the usual conditions of acid hydrolysis, when it was consistently lower. This difference was recognized by Gale (1945), who applied a correction factor of +5% for racemization during hydrolysis (Tristram, 1948).

Table 2. *Recovery of glutamic acid from control mixtures on a column of Amberlite IR-4B*

Control mixture	Glutamic acid N (mg.) Taken	Found	Recovery (%)
A	1·155	1·154	99·9
A	1·155	1·156	100·1
A	1·155	1·184	102·6
B	3·954	3·939	99·6
B	3·954	3·961	100·2
		Mean	**100·5**
		S.E.±	0·9

The results of different workers on the extent of racemization of glutamic acid under the conditions of protein hydrolysis are conflicting. Arnow & Opsahl (1940), Johnson (1940*b*), Jeannerat (1942), Klingmuller (1943) and Wieland & Paul (1944) observed a fall in optical rotation when pure

Table 3. *Recovery of* L-*glutamic acid from control mixtures by the enzymic method*

Mixture and treatment	No. of experiments	% Recovered (Mean ± S.E.)	% D-isomer by difference (Mean ± S.E.)
(*a*) L-Glutamic acid hydrochloride alone	10	99·7 ± 0·4	0·3 ± 0·4
(*b*) L-Glutamic acid hydrochloride + L-aspartic acid	4	99·3 ± 0·5	0·7 ± 0·8
(*c*) Control mixture *A**	4	97·8 ± 1·2	2·2 ± 1·0
(*d*) L-Glutamic acid hydrochloride refluxed 24 hr. in 6 N-HCl and taken to dryness several times *in vacuo*	8	94·7 ± 0·5	5·3 ± 0·6
(*e*) Control mixture *A** treated as (*d*)	10	94·9 ± 0·7	5·1 ± 0·8
(*f*) Control mixture *C** treated as (*d*)	8	94·7 ± 0·7	5·3 ± 0·9

* Table 1.

Table 4. *Racemization of* L-*glutamic acid under the conditions of protein hydrolysis*

Treatment	No. of experiments	Mean $[\alpha]_D^{20}$ (2·5 N-HCl, $c = 4$)	% D-isomer
(*a*) None	5	+31·43	—
(*b*) Solution concentrated to dryness once at 40°	2	+31·67	—
(*c*) Solution concentrated to dryness several times at 40°	3	+30·78	1·3
(*d*) Refluxed 24 hr. in 6 N-HCl and taken to dryness once at 40°	2	+29·72	3·0
(*e*) As (*d*) but taken to dryness several times at 40°	6	+28·54	4·9

L-glutamic acid was heated with HCl. On the other hand, Kögl (1949) detected no change in rotation on treating L-glutamic acid with 10 N-HCl for 24 hr. at either 115 or 130°.

Glutamic acid isolated from normal proteins after acid hydrolysis by Johnson (1940*a*), Chibnall, Rees & Williams (1943), Klingmuller (1943) and Wieland & Paul (1944) appeared to contain a small proportion of D-isomer as measured by the optical rotation. DL-Glutamic acid labelled with ^{15}N was added to a protein hydrolysate by Graff, Rittenberg & Foster (1940). From the isotope ratio in the L- and DL-glutamic acid isolated it was concluded that there was no D-isomer in the hydrolysate.

The present polarimetric results with L-glutamic acid (Table 4) confirm that racemization occurs during refluxing with 6 N-HCl for 24 hr. and also when the usual technique is applied to remove the excess mineral acid. Under normal conditions applicable to protein hydrolysates (Table 4*e*) there is a decrease in the specific rotation which might result from destruction of 10% of the L-isomer or the inversion of 5% of it. The latter explanation would appear the more probable from the parallel loss found with the decarboxylase procedure (Table 3*d*, *e*, *f*). It is to be remembered, however, that the small loss recorded during the removal of excess mineral acid, observed with L-glutamic acid itself (Table 4*c*) might be due to anhydride formation (Wilson & Cannan, 1937), or perhaps to inversion during the closing or opening of the anhydride ring. A sample of L-pyrrolidonecarboxylic acid was accordingly passed through the usual hydrolysis procedure and, after removal of the excess mineral acid, 86% of the expected glutamic acid hydrochloride was crystallized out by saturating the aqueous solution with hydrogen chloride and allowing to stand at 0° for 3 days. The anhydride used had $[\alpha]_D^{20}$ −9·92°; Foreman (1914) quotes −11·4°, and Abderhalden & Kautzsch (1910) −10·8° and −11·5° for the L-isomer and Ratner (1944) +11·4° for the D-isomer. Accepting the higher figure, the present preparation contained a maximum of 6% of the D-isomer. The isolated glutamic acid hydrochloride crystals ($[\alpha]_D^{20}$ +29·8° in 2·5 N-HCl) contained about 3% of D-isomer and accounted for 86% of the pyrrolidonecarboxylic acid. If inversion does indeed occur during the reversible formation of the pyrrolidone ring, it is limited in extent, and would not make a large contribution to the total racemization observed under the conditions of protein hydrolysis.

RESULTS AND DISCUSSION

The experiments described above suggest that during the normal conditions of acid hydrolysis adopted in this laboratory about 5% inversion of L-glutamic acid occurs. On such a supposition the data given in Table 5 suggest that no significant amount of D-glutamic acid residues was present in the proteins concerned. Many replicate analyses were made in each case, and on statistical examination of the results it was found that individual estimates by the decarboxylase method were accurate to ± 5% and by the Amberlite method to ± 2%.

Protein preparations reported to contain large amounts of the D-isomer have been analysed in order to explore the application of the differential procedure to such material (Table 6). A significant proportion was found in the cells of *Lactobacillus casei*, confirming Camien, Salle & Dunn (1945), but not in tobacco-mosaic-virus protein, in contrast to the finding of Lewis & Olcott (1945), who used a different strain. All the products prepared by alkali treatment of casein contained, as was ex-

Table 5. *Isomers of glutamic acid in hydrolysates of purified proteins*

(Glutamic acid N as % total N.)

Protein	Both isomers Mean S.E.	L-Isomer Mean S.E.	D-Isomer (by diff.) Mean S.E.*	D-Isomer as % total glutamic acid
Edestin	9·83±0·2	9·60±0·2	0·23±0·27	2·4†
Horse myoglobin	9·33±0·2	8·71±0·3	0·62±0·22	6·6
Beef insulin	10·51±0·3	10·03±0·1	0·48±0·14	4·6

* S.E. of the difference between means in columns 1 and 2.
† Not significant.

Table 6. *Isomers of glutamic acid in hydrolysates of protein products reported to yield* D-*glutamic acid*

(Glutamic acid N as % total N.)

Product	Both isomers Mean	L-Isomer Mean S.E.	D-Isomer (by diff.)	D-Isomer as % total glutamic acid
Tobacco-mosaic-virus protein	7·38	7·05±0·22	0·33	4·5*
Cells of *Lactobacillus casei*	5·62	4·77±0·05	0·85	15·1
Alkali-treated proteins				
Casein	14·27	7·95±0·27	6·32	44·3
Caseose	11·90	6·57±0·11	5·33	44·8
Gelatin protein fraction	6·12	4·21†	1·91	31·2
Gelatin peptide fraction	5·29	3·38†	1·91	36·0

* Not significant.
† Insufficient estimates for statistical analysis.

pected from the work of Dakin & Dudley (1913), large amounts of the D-isomer. The results with gelatin are contrary to those reported by Dakin (1912) since here too the glutamic acid was largely racemized.

SUMMARY

1. The L- and total glutamic acid were estimated in three purified proteins and in protein preparations.

2. Five per cent of D-isomer was produced from L-glutamic acid under the conditions of acid hydrolysis and subsequent treatment of the hydrolysate for analysis.

3. D-Glutamic acid in excess of that estimated to be formed by inversion was found in hydrolysates of proteins which had been treated with dilute alkali, and in the cells of *Lactobacillus casei*, but not in four purified plant and animal proteins or in tobacco-mosaic-virus protein.

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